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Sent: Tuesday, September 10, 2002 12:00 PM
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Please provide the following references for use in examining 09/532,001

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Thank-You!

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In Vivo Gene Transfer and Gene Modulation in Hypertension Research

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Abstract Transgenic technologies and homologous recombination approaches have been useful for studying the roles of specific genes in systemic hypertension. Recently, we and others have introduced the use of in vivo gene transfer to study the effects of local gene overexpression or inactivation in hypertension. Using in vivo gene transfer for the blood vessel, we have documented the direct hypertrophic action of local angiotensin and the growth-inhibitory effect of nitric oxide. In vivo gene transfer is also an effective method for discovering the unknown functions of a newly cloned gene. Using this approach, we identified the in vivo growth-inhibitory action of the angiotensin II type 2 receptor. In addition, we have developed a novel strategy using transcriptional factor "decoy" oligonucleotides to regulate

the interaction of *cis*- and *trans*-acting factors involved in the modulation of gene expression in vivo. Thus, the decoy approach can "switch" on or off specific genes in selective tissues in vivo, thereby influencing local gene expression and tissue function. For example, using decoy oligonucleotides, we have "turned on" renin gene expression in the rat liver, in which it is usually not expressed, resulting in increased hepatic and plasma renin levels. Thus, in vivo gene transfer technology provides us with a new tool for in vivo characterization of genes involved in hypertension that has potential application in human therapy. (*Hypertension*. 1996;28:1132-1137.)

Key Words • gene transfer • hypertension, essential • genetics

Essential hypertension is a common disease and a major cause of cardiovascular morbidity and mortality. Genetics constitutes an important component of the pathogenesis of essential hypertension; however, the specific genes responsible for hypertension have not been clearly identified. We recently reviewed the current approaches to the study of the genetics of complex cardiovascular diseases such as hypertension (Table 1).¹ Gene mapping techniques using inbred hypertensive rat and mouse strains have resulted in genetic loci identification, but the specific culprit genes at these loci have not been cloned. Many known genes have also been proposed as candidates responsible for genetic hypertension. With the recent advent of molecular genetic technologies such as transgenic methodology and the techniques of homologous recombination, we now can study the contribution of specific candidate genes to changes in blood pressure. Thus, one can alter the genotype of an animal and study the resultant effects on the phenotype. For example, Mullins et al² reported that the overexpression of the mouse *Ren-2* renin gene resulted in the development of a transgenic rat strain with fulminating hypertension. Kim et al³ produced mice carrying 0, 1, 2, 3, or 4 functional copies of the murine wild-type angiotensinogen gene at its normal chromosomal location and observed that the blood pressure of these transgenic mice showed significant and almost linear increases according to the number of copies of the angiotensinogen gene that a mouse carried. Recently, we used gene targeting to disrupt the gene encoding the AT₂ receptor and

demonstrated that the "knockout" mice exhibit enhanced Ang II-mediated blood pressure elevation.^{4,5} These powerful techniques, useful in examining the genetic mechanism of hypertension, have some limitations. In transgenic studies that use an expression plasmid driven by a constitutive promoter, the effect of the overexpressed transgene is exerted throughout development. Without the use of a tissue-specific promoter, it would be difficult to define the contribution of a gene that is expressed only in selective tissue or tissues. Furthermore, the temporal sequence of the endogenous expression of a specific gene and its potential aberrancy in hypertension may be difficult to capture. In the case of gene targeting, if disruption of the candidate gene is lethal and/or prohibits germ line transmission, one may not be able to study the role of that candidate gene in hypertension.

Our laboratory has been developing a complementary approach to the transgenic technique involving in vivo gene transfer to study the contribution of specific genes to cardiovascular diseases. The potential usefulness of this approach is summarized in Table 1. This approach may be an effective method for study of the local function of target genes. In vivo gene transfer can produce the local overexpression of a target gene (gain-of-function approach) or the inhibition of a specific gene (loss-of-function approach). Gene transfer can theoretically be performed at the desirable age of the animal, barring technical issues. Thus, the roles of candidate genes that are activated or inactivated during the pathogenesis of hypertension can be explored with this approach. In vivo gene transfer is also an effective method for study of the unknown functions of a newly cloned gene. In addition, we have recently developed a novel approach using transcriptional factor "decoy" in vivo to modulate the interaction of a specific transcription factor with the corresponding *cis*-element that is involved in tissue-specific gene expression. However, in vivo gene transfer has certain limitations, including the relative low efficiency of transfection, the limited duration of transgene expression,

Received June 16, 1996; first decision July 29, 1996; revision accepted August 15, 1996.

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Selected Abbreviations and Acronyms

Ang II = angiotensin II
 AT₁, AT₂ = angiotensin type 1, type 2 (receptor)
 CAT = chloramphenicol acetyltransferase
 HVJ = hemagglutinating virus of Japan
 NO = nitric oxide
 NRE = negative regulatory element
 NREB = negative regulatory element binding protein
 SHR = spontaneously hypertensive rats(s)
 VSMC = vascular smooth muscle cell

the technical difficulty in studying embryonic and developmental influences of the target gene, and the limited ability to study simultaneous gene expression or inhibition in multiple tissues. Nevertheless, the simplicity of the technology, the possibility of activating or inhibiting the expression of a gene at a specific time and in a specific tissue, and the ability to examine the local effects of a gene independent of systemic effects are some of the advantages of this approach. Thus, this technology is a useful addition to the existing molecular genetic methods in the study of genes in cardiovascular diseases. In this article, we review the current experience with this strategy for genetic studies of hypertension.

Principles of Gene Transfer for Hypertension Research

Table 2 summarizes in vivo gene transfer in hypertension research.

Gain-of-Function Approach

The gain-of-function approach examines the effect or effects of overexpressing a specific gene to elucidate the function or role of that gene and/or to develop a therapeutic strategy based on the overproduction of the specific gene product. In the cardiovascular system, many factors have been postulated to exert autocrine-paracrine actions. Definitive proof for their local functions cannot be obtained because in vivo studies are limited by the multiplicity of coexisting variables, the difficulties in manipulating individual components, and the methodological limitations in studying the function of locally produced modulators in the absence of any contribution by the circulation. Local overexpression of a candidate gene will give us insight into the contribution of that gene to local tissue function in hypertension. Using this approach, we have documented the direct vascular hypertrophic action of Ang II⁶ and the growth-inhibitory effect of NO.¹⁷ Fur-

TABLE 2. In Vivo Gene Transfer in Hypertension: Summarized Research

Tissue	Gene	Reference
Gain-of-function approach		
Carotid artery	ACE	6
	ec-NOS	17
	AT ₂ receptor	39
Loss-of-function approach (antisense)		
Carotid artery	cdc2 kinase, PCNA	9
	cdk2 kinase	19
	cdc2 kinase, PCNA	12
Vein graft	Angiotensinogen	16
Liver	Angiotensinogen	43
Decoy approach		
Submandibular gland, kidney	Renin	42
Carotid artery	E2F	11
Liver	Renin	10
Liver	Angiotensinogen	44

ACE indicates angiotensin-converting enzyme; ec-NOS, endothelial cell NO synthase; PCNA, proliferating cell nuclear antigen; cdc2 kinase, cell division cycle 2 kinase; and cdk2 kinase, cyclin-dependent kinase 2.

thermore, the overexpression of NO synthase has been shown to be therapeutically effective in a model of experimental vascular proliferative disease.

Loss-of-Function Approach

Another strategy involves an antigene approach in which targeted genes are inactivated by specific antisense DNA or RNA that is either synthesized as oligonucleotides or transcribed from an expression plasmid. The antisense hybridizes specifically with its target gene or mRNA, thereby inactivating gene transcription or translation. Antisense oligodeoxynucleotides have been used successfully to inhibit specific protein synthesis in a number of biological systems.^{8,18} Antisense technology can be applied to any candidate gene whose nucleotide sequence is known. The effectiveness of antisense oligonucleotides is limited by the low efficiency of cellular uptake and subsequent rapid degradation of the oligonucleotide in the endocytotic-lysosomal pathway. Modification of the oligonucleotide backbone such as phosphorothioate linkages and transfection with the HVJ liposome have enhanced stability and efficiency.^{9,19} The use of antisense oligonucleotides as a strategy to selectively block the expression of specific genes represents an important innovation in cell biology research, and this paradigm of gene inhibition has many potential therapeutic applications.

TABLE 1. Comparison of In Vivo Gene Manipulation Methodologies

Parameter	Transgenic Animals	Gene Disruption	Gene Transfer
Stable genomic changes	Yes	Yes	Usually not
Time of effect	Usually throughout development (can be regulated)	Usually throughout (can be regulated)	Usually adult
Duration of effect	Usually permanent (can be regulated)	Usually permanent (can be regulated)	Usually transient
Location of effect	Usually systemic (can be tissue specific)	Usually systemic (can be tissue specific)	Local/systemic
Species	Mouse/rat (rabbit)	Mouse (rat)	No limitation
Cost	Expensive	Expensive	Inexpensive
Time to produce	Long	Long	Short

Adapted from Dzau et al¹ with permission.

In Vivo Modulation of Gene Transcription

In vivo gene transfer techniques may be useful for the study of promoter function in vivo. Previous investigators have reported the feasibility of direct gene transfer into the heart or arterial wall for characterization of the sequences necessary for proper gene regulation in vivo.²⁰⁻²² We have used the transcriptional factor "decoy" method to test the validity of specific *cis*- and *trans*-acting factor interaction involved in tissue-specific gene expression in vivo. This strategy is based on the competition for a *trans*-acting factor between the endogenous *cis*-element present in a target gene and an exogenously added oligonucleotide corresponding to that *cis*-sequence. This approach should prevent the *trans*-acting factor from interacting with the endogenous *cis*-element, thereby modulating gene expression. Bielinska et al²³ used this method to block octamer transcription factor- or nuclear factor- κ B-dependent *trans*-activation in B lymphocytes. Sullenger et al²⁴ reported that overexpression of the sequences containing *trans*-activation response element rendered CD4-positive human T lymphoid cells that were resistant to human immunodeficiency virus replication. Thus, using the decoy approach, one can turn off or on a target gene and thereby investigate the function of the target gene in vivo.

Potential Gene Therapy

Molecular and cellular biology research has identified critical mediators that may be involved in hypertension. The capacity to manipulate the gene expression of these mediators with antigene or gene augmentation strategies may be applied toward future gene therapy of hypertension. However, current in vivo methods for gene transfer are still limited by relatively low transfection efficiency and their potential toxicity.

Methods of In Vivo Gene Transfer

Virus-Mediated Gene Transfer

The feasibility of transfecting blood vessels with foreign DNA in vivo was first reported with the use of retroviral vectors.^{25,26} However, this approach yielded limited transfection efficiency. The adenoviral vector has been used with improved efficiency for gene transfer into blood vessels.^{27,28} Recently, Ohno et al²⁹ demonstrated that adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene into injured porcine arteries, accompanied by administration of ganciclovir, resulted in the destruction of proliferating VSMCs and consequently the inhibition of neointimal hyperplasia. Localized arterial infection of replication-defective adenoviral vector containing an expression plasmid encoding a nonphosphorylatable, constitutively active form of retinoblastoma gene product significantly reduced VSMC proliferation and neointima formation in both the rat carotid and porcine femoral artery models of restenosis.³⁰ These viral vectors have established efficiency for gene transfer but are limited by their potential toxicity. Retrovirus-mediated transfer allows genomic integration but carries the risk of oncogene activation and viral transformation. Adenoviral vectors have been reported to stimulate immunological and cytotoxic responses. Modification of these viral vectors is under way to enhance their efficiency and minimize their toxicity for clinical application.

Fusigenic Viral Liposome-Mediated Gene Transfer

We have recently used a highly efficient and simple method of DNA transfer with fusigenic viral liposome complex. This method involves the encapsulation of DNA in neutral liposomes that are then complexed with UV-inactivated Sendai virus (also known as HVJ).³¹ The HVJ envelope fusion proteins enhance the fusion of the liposomes with cell membranes, facilitating the intracellular delivery of the DNA. A further modification, complexing the DNA with nonhistone protein, ie, high mobility group-1 protein, increases the nuclear translocation of the DNA and the transcription of the transgene. The HVJ liposome technique possesses improved properties for gene transfer, such as efficiency, safety, simplicity, and brevity of incubation time as well as no limitation of inserted DNA size; furthermore, it has been shown to be efficient in multiple animal species. This method is also suitable for the transfer of oligonucleotides, whereas the other virus-mediated transfer techniques are not.

Liposome-Mediated and Mechanically Mediated Gene Transfer

Many investigators have reported the utility of cationic liposomes for transfection in vitro. Although Lim et al³² reported the successful transfection of DNA using liposomes into intact coronary and peripheral arteries in vivo, this method requires a long incubation time, and the transfection efficiency is low. Indolli et al³³ demonstrated that the transfection of dominant-negative *ras* gene with pluronic gel prevented neointimal formation in balloon-injured rat carotid artery. Mechanical DNA transfection with high-energy microparticle bombardment³⁴ appears to be an effective method for in situ gene transfer into solid organs such as the liver. Other methods of mechanical DNA transfer, eg, transduction under increased pressure, are currently under investigation.

In Vivo Gene Transfer in Hypertension

Autocrine-Paracrine Factors in Vascular Remodeling

The pathogenesis of hypertension involves a process of vascular remodeling associated with increased local expression of biologically active substances that are postulated to play pathophysiological roles. In vivo gene transfer technology provides the capability of defining the roles of these genes. In hypertension, the arteries undergo a process of vascular hypertrophy. The potential role of angiotensin in vascular growth has been suggested from indirect evidence derived from in vitro cell culture studies, the infusion of angiotensin, and the administration of pharmacological inhibitors of the renin-angiotensin system in vivo. To circumvent the contributions of hemodynamic and neurohumoral effects, we applied gene transfer methods to examine the consequence of local overproduction of angiotensin in a segment of the carotid artery in the intact rat. This approach is particularly powerful because the locally transfected vascular segment and the nontransfected vessels are exposed to the same hemodynamic and circulating factors. We used HVJ liposome-mediated transfer of an angiotensin-converting enzyme expression vector into intact rat carotid artery to increase local expression of vascular angiotensin-converting enzyme. Our results demonstrated that increased local expression of angiotensin-converting enzyme within the vessel wall pro-

motes autocrine-paracrine Ang II-mediated vascular hypertrophy in vivo.⁶

Endothelium-derived NO, another important vasoactive substance, has been postulated to be an important endogenous inhibitor of vascular lesion formation.³⁵ In vitro studies have demonstrated the ability of NO to inhibit VSMC growth and migration.³⁶ In vivo administration of NO donors has been shown to inhibit neointimal hyperplasia.^{36,37} However, the interpretation of the in vivo results with NO donors is clouded by the hemodynamic effects of these agents. Accordingly, we overexpressed endothelial cell NO synthase (ec-NOS) gene in the balloon-injured rat carotid artery model, which is characterized by neointimal hyperplasia involving VSMC migration and proliferation in vivo. In vivo transfection of ec-NOS cDNA restored NO production in the injured, endothelium-denuded rat carotid artery, resulting in a marked reduction (70%) of neointimal lesion formation.¹⁷ These findings provide direct evidence that NO is an endogenous inhibitor of VSMC growth and support the importance of NO in modulating the vascular remodeling in hypertension.

In Vivo Functions of Newly Cloned Genes in Hypertension

Another application of in vivo gene transfer is to examine the unknown function or functions of a novel molecule. Most of the known effects of Ang II in adult tissues are mediated by the AT₁ receptor. Recently, a second receptor subtype known as the AT₂ receptor was cloned^{7,38}; however, little is known about the regulation and physiological functions of this novel receptor. The highly abundant expression of this receptor during embryonic growth and its rapid disappearance after birth have led to the suggestion that this receptor is involved in growth, development, and/or differentiation. With a gain-of-function approach using in vivo gene transfer, we tested the hypothesis that the AT₂ receptor can modulate VSMC growth. We observed that in vivo gene transfer and the overexpression of the rat AT₂ receptor in injured rat carotid artery resulted in decreased DNA synthesis and the attenuation of neointimal hyperplasia.³⁹ When neointimal area was analyzed at 14 days after injury and transfection, a 70% decrease was observed. Taken together, these results suggest that the AT₂ receptor exerts an antiproliferative effect on cell growth.⁴⁰ Additional data demonstrated that the AT₂ receptor action counteracted the growth-promoting activity of the AT₁ receptor. These observations have important implications in our understanding of the role of locally generated vasoactive substances in the process of vascular remodeling. Furthermore, these results demonstrate the usefulness of this approach for identifying the unknown functions of newly cloned genes in hypertension.

Gene Transcription In Vivo

Renin, an aspartyl proteinase involved in the regulation of cardiovascular homeostasis, exhibits tissue-specific regulation. The DBA/2J mouse contains two renin gene loci: *Ren-1^d* and *Ren-2^d*. *Ren-2^d* but not *Ren-1^d* is expressed in submandibular gland, and both are expressed in the kidney. Our previous in vitro studies of the molecular mechanisms of mouse renin gene expression using promoter-reporter gene experiments and gel mobility shift assays suggest that tissue-regulated expression depends on the in-

teraction of an NRE in the renin gene promoter with the transcriptional factor NREB.^{13,41} These data are derived from experiments with in vitro biochemical methods and noncognate cell systems and need to be verified in vivo. We examined the molecular mechanism at the in vivo level using direct gene transfer.⁴² Fragments of the *Ren-1^d* or *Ren-2^d* promoter were fused to a CAT gene expression vector. These constructs complexed in HVJ liposome were injected directly into the mouse submandibular gland or intra-arterially into the mouse kidney via the renal artery. In the submandibular gland, *Ren-1^d* fragment containing the NRE abolished CAT expression, and deletion of the NRE restored CAT expression. The homologous fragment from the *Ren-2^d* promoter did not inhibit CAT expression, and deletion of the 150-bp insertion resulted in inhibition. Cotransfection of *Ren-1^d* construct with *Ren-1^d*-NRE oligonucleotides as transcriptional factor decoy restored CAT expression. Contrary to the situation in the submandibular gland, transfection with a *Ren-1^d* fragment-CAT construct or *Ren-2^d* fragment-CAT construct into the kidney showed similar levels of CAT expression.

We have also used in vivo DNA transfection to study the interaction of a specific DNA element with nuclear protein and to modulate gene transcription in vivo. We used the transcriptional factor decoy approach to investigate renin gene expression. Renin is synthesized in high quantities in the juxtaglomerular cells of the kidney, but little or none exists in the liver. We have identified by sequence homology analysis that the mouse, rat, and human renin genes contain the conserved NRE.^{14,15} We examined the possibility that the interaction between the NRE in the promoter region of the rat renin gene and the NREB in the liver contributes to the suppressed hepatic renin gene expression in vivo. We used in vivo transfection of NRE transcriptional factor decoy double-stranded oligodeoxynucleotide into the rat liver via portal vein infusion.¹⁰ Gel mobility shift assay showed that transfected NRE decoy blocked endogenous NREB binding with NRE in the rat renin gene. "Turning on" the renin gene in the rat liver by blocking NREB binding resulted in increased hepatic renin expression and circulating renin activity. Taken together, these results document the importance of NREB in the inhibition of renin gene expression in the rat liver in vivo and suggest the possibility of in vivo renin gene modulation by the transcriptional factor decoy approach. These results also revealed that the in vivo transfer technique with the HVJ liposome combined with the transcriptional factor decoy is useful for the general investigation of *cis*- and *trans*-element interactions in tissue-specific gene regulation in vivo.

Applications Toward Gene and Molecular Therapy

We have reviewed previously the potentials of in vivo gene transfer for gene therapy of cardiovascular diseases.¹ Our laboratory has demonstrated the effectiveness of antisense oligonucleotide to cell cycle regulatory genes,^{9,10} decoy oligodeoxynucleotide to transcriptional factor E2F,¹¹ and expression plasmid encoding ec-NOS in the treatment of experimental models of vascular proliferative disease in rat¹⁷ and in the development of genetically engineered vascular graft ex vivo.¹² In vivo gene transfer strategy may also be applied toward hypertension therapy.

We have applied the HVJ liposome method to transfect antisense oligodeoxynucleotides, and this method enhanced the efficiency and prolonged the half-life of anti-

sense oligodeoxynucleotides *in vitro* and *in vivo*.^{9,19} Using this delivery system, Tomita et al¹⁶ transfected antisense oligodeoxynucleotide against rat angiotensinogen into the rat liver via portal vein infusion and observed that antisense oligodeoxynucleotide transfection resulted in a transient decrease (up to 7 days after transfection) in plasma angiotensinogen levels in the SHR, consistent with the reduction of hepatic angiotensinogen mRNA. Plasma Ang II concentration was also decreased in rats transfected with antisense oligodeoxynucleotides, and transient decreases in blood pressure from days 1 to 4 were observed.

The brain has all the components of an Ang II production system, and an overactive brain local renin-angiotensin system has been implicated in the development and maintenance of high blood pressure in the SHR. To examine whether angiotensinogen reduction in the brain would lower blood pressure in the SHR, Wielbo et al⁴³ administered phosphorothioated antisense oligonucleotide targeted to angiotensinogen mRNA intracerebroventricularly in SHR. They demonstrated that intracerebroventricular administration of antisense oligonucleotide for angiotensinogen lowered blood pressure to normotensive levels in the SHR. They also reported that angiotensinogen production was significantly decreased in the brain stem and hypothalamus of the rats treated with intracerebroventricular antisense oligonucleotide. On the other hand, intrarterial antisense oligonucleotide against angiotensinogen did not affect blood pressure.

Recently, Morishita et al⁴⁴ studied the effectiveness of the transcriptional factor decoy approach in lowering blood pressure in the SHR. Using HVJ liposome as the delivery system, they infused into the portal vein of SHR double-stranded oligodeoxynucleotides against angiotensinogen gene-activating elements (AGE) 2 and 3, which are present in the promoter region. They demonstrated that decoy oligodeoxynucleotides against AGE2 infusion decreased hepatic angiotensinogen mRNA and plasma Ang II concentration. In parallel, systolic pressure also decreased from day 1 to day 6 after decoy oligodeoxynucleotide treatment, whereas AGE3 decoy had little effect. Taken together, these studies demonstrate the feasibility of specific antigene strategy for the treatment of hypertension.

In hypertension drug therapy, many effective agents with minimal side effects already exist. Therefore, the current application of DNA transfer provides primarily a proof of concept for these genetic and molecular principles. However, it may pave the way for future molecular therapeutics aimed at blocking the fundamental pathophysiological and genetic mechanisms of hypertension. As technology for gene transfer improves, thereby enabling stable genomic integration with long-term *in vivo* gene expression in the absence of any side effects, one can envision using gene replacement, gene augmentation, or gene inhibition *in vivo* to correct specific genetic defects in human hypertension in the next millennium.

Conclusion

For hypertension research, *in vivo* gene transfer appears to be a useful technology for defining the role of specific genes in the pathogenesis of hypertension. It may be useful for potential gene therapy in hypertension. However, this technology is in its infancy. Future research in improving the methodologies should facilitate the widespread appli-

cation of this strategy for hypertension research and therapy.

Acknowledgments

This work was supported by National Institutes of Health (NIH) grants HL-46631, HL-35252, HL-35610, HL-48638, and HL-07708 and the American Heart Association Bugher Foundation Center for Molecular Biology in the Cardiovascular System as well as grants from CIBA-Geigy, Basel, Switzerland, and from the SmithKline Beecham Research Foundation. Victor J. Dzau is the recipient of NIH Merit Award HL-35610.

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